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Protease Bypass of Temperature-Sensitive Murine Leukemia Virus Maturation Mutants

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Cells infected with certain temperature-sensitive mutants of Moloney murine leukemia virus synthesize the virion precursor proteins but neither bud virions nor cleave precursor proteins to their mature form. Addition of proteases to cells infected with these mutants caused cleavage of the precursor proteins $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ to their mature forms at the nonpermissive temperature. Concomitantly there was release from the cells of morphologically normal virions. The enzymatically inactive $Pr180^{gag\text{-}pol}$ was cleaved to active reverse transcriptase (p85), which was found in the released particles. External protease treatment apparently bypassed the lesion in these viral mutants, suggesting that their defect may involve a virus-specific protease.

Murine leukemia viruses (MuLV) can be grown in fibroblast or lymphocyte tissue culture lines where the virus is expressed chronically and indefinitely without impairing cell growth (for review, see references 4 and 11). The translation of the viral mRNAs produces polyprotein precursors, which must undergo proteolytic cleavages to be transformed into the mature virion proteins (for review, see reference 11). The cleavage steps occur mainly in precursors that are associated with the plasma membrane of the infected cells (21). It is at the plasma membrane that *gag*, *pol*, and *env* proteins assemble with virion RNA into a viral bud and eventually into a complete virion (7, 18).

In chronically infected cell populations, the assembly process is too asynchronous to study biochemically or morphologically. Our understanding of retroviral maturation has therefore come largely from an analysis of mutants conditionally blocked in protein processing and virion morphogenesis. Rauscher *ts*24 (R-*ts*24) and Moloney *ts*3 (M-*ts*3) are the prototypes of such MuLV mutants (12, 20). R-*ts*24, isolated after nitrosoguanidine mutagenesis, and M-*ts*3, isolated as a spontaneous mutant, have virtually indistinguishable phenotypes (18). Cells infected with these mutants and maintained at the nonpermissive temperature (39°C) release virtually no virions whether measured by assaying the culture media for reverse transcriptase activity, infectious virus, or viral proteins.

When cells infected by R-*ts*24 or M-*ts*3 are metabolically labeled at 39°C with [^{35}S]methionine and cellular lysates are analyzed electrophoretically, an intracellular accumulation of *gag* and *gag-pol* precursors is seen (13, 14, 18). The maturation block is very rapidly reversed upon shifting the cultures to 32°C; prelabeled precursors are then detected in extracellular particles as mature virion proteins (18). Reverse transcriptase activity is also found extracellularly, and supernatant fluids are infectious. In fact, the work of previous investigators and of this laboratory has confirmed that harvests collected only 30 min after temperature shift are infectious (7; P. Traktman, unpublished results). In addition, the burst after the shifting of cultures to 32°C is fully induced even in the presence of cycloheximide, a potent inhibitor of protein synthesis (7, 18; P. Traktman, unpublished results). The recovery of normal virus maturation can apparently proceed solely with components synthesized at 39°C. To date, no agreement has been reached on the identity of the lesion in these mutants. Although a defect in one of the precursors which precludes maturation has been suggested, the defect may as well reside in the cleavage itself, perhaps in a possible viral protease. In this paper, we present evidence that the addition of extracellular protease at 39°C to these infected cells causes a rapid release of the block in viral assembly.

MATERIALS AND METHODS

Viruses and cells. R-*ts*24 was supplied by S. Aaronson, National Cancer Institute. The viruses were grown in NIH/3T3 cells, usually in cell lines derived by low-multiplicity infection and immediate cloning. To construct such clones, NIH/3T3 cells were infected

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with the virus at a multiplicity of 0.3 and then seeded in 96-well microtiter trays at a density of 0.3 cells per well. Infected clones were identified by assaying the culture medium for reverse transcriptase activity, infectious virus, or viral proteins. Two of these clonal lines, 3N7 and 24N3, have been previously described (18). The cells were maintained in Dulbecco modified Eagle medium (DME) supplemented with 10% calf serum and buffered with CO_2 and were passaged at 37°C except where noted in the text.

Titers of infectious virus were determined by the XC plaque assay (10). Assays were performed at 32, 37, or 39°C as necessary.

All viral infections were done in the presence of 8 μg of Polybrene per ml (Aldrich Chemical Co.) to enhance adsorption. Polybrene was sometimes included in the growth medium for several days at 2 μg/ml to enhance viral spread. Viral harvests were collected 12 to 18 h after medium change, except where otherwise indicated, and filtered through 0.45-μm filters before use.

Metabolic labeling. For metabolic labeling of cell proteins, monolayers were washed twice with phosphate-buffered saline (PBS) and labeled with [^{35}S]methionine (New England Nuclear Corp.) in serum-free DME lacking methionine. Labelings were routinely performed with 50 μCi/ml and varied in duration from 40 min to 4 h, as described below. Chases were performed by adding prewarmed complete DME plus serum, with or without removal of the labeling medium.

For preparation of overnight harvests of virus with metabolically labeled proteins, monolayers in 10-cm dishes were washed and then labeled with 125 μCi of [^{35}S]methionine in 4 ml of methionine-free DME supplemented with 2% DME and 10% dialyzed fetal calf serum. The medium was collected after 10 to 16 h and processed as described below.

Virus purification. Culture fluids from virus-infected lines were collected and filtered through 0.45-μm filters or centrifuged at 1,200 rpm for 10 min to remove cells. The virus was then layered on discontinuous gradients of 25 and 45% sucrose in PBS. After ultracentrifugation for 2.5 to 3 h at 22,000 to 30,000 rpm, the interface was collected and diluted with PBS, and the virus subsequently was collected by sedimentation at 38,000 to 45,000 rpm for 1 to 3 h.

Reverse transcriptase assay. Supernatant fluids were either assayed directly or first concentrated (typically 40-fold) by ultracentrifugation at 38,000 to 45,000 rpm for 1 to 2 h. Virus pellets were suspended in 20 mM Tris (pH 8.3) and disrupted with detergent, and the exogenous activity was assayed by using a polyriboadenylate template and oligodeoxythymidylate primer as described (8). Enzyme activity was quantitated by measuring incorporation of [^{3}H]TTP into trichloroacetic acid-precipitable material.

Protease treatment of intact cells. Subconfluent cultures of infected NIH/3T3 cells were rinsed twice with DME and then treated with proteases appropriately diluted in 2 to 2.5 ml of DME (or, less frequently, in Hanks balanced salt solution). Several preparations of trypsin were used: partially purified hog pancreas extract used for passaging tissue culture cells (GIBCO Laboratories), trypsin-tolylsulfonyl phenyalanyl chloromethyl ketone (Worthington Diagnostics), and 3X crystallized trypsin (Worthington). Thermolysin (Calbiochem), papain (Sigma Chemical Co.), plasmin (Sigma), and α-chymotrypsin (Worthington) were also used. The concentrations (routinely 2 to 50 μg/ml) and duration of protease treatment are described below. Reactions were stopped by rapid chilling and the addition of one of the following: *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; Calbiochem), phenylmethylsulfonyl fluoride (Sigma), soybean trypsin inhibitor (Calbiochem), bovine serum albumin (Pentex, Miles Laboratories), or calf serum. After treatment, cells were usually detached or only loosely adherent and easily removable from the culture dishes. Control cells were gently scraped from the culture dishes. For further analysis, cells and fluid were either analyzed together or first separated by centrifugation at 2,000 rpm for 10 min at 4°C.

Immune precipitations. To analyze the proteins of metabolically labeled cells, cultures were lysed on ice in a lysis buffer containing sodium dodecyl sulfate, deoxycholate, and Triton X-100 as previously described (18). Cell lysates, prepared at 10^6 cells per ml, were clarified by centrifugation at 1,200 rpm for 10 min and then precleared by incubation with normal serum and fixed *Staphylococcus aureus* (The Enzyme Center, Tufts Medical Center) followed by a 1- to 3-h centrifugation at 35,000 to 45,000 rpm at 4°C (5). ^{35}S-labeled virions were lysed with the appropriate concentration of the same phospholysis buffer to give the same final conditions.

Samples were immunoprecipitated with 1 to 5 μl of the appropriate antiserum overnight at 4°C. Sera were either previously prepared in this laboratory (18) or were kindly supplied by Roger Wilsnack, National Cancer Institute. Fixed *S. aureus* (25 μl of a 10% stock) was then added, and incubation was allowed to proceed for at least 1 h on ice (5). The immune precipitates were collected by centrifugation and washed twice with lysis buffer. The final *S. aureus* pellets were suspended in sample buffer, containing sodium dodecyl sulfate, 2-mercaptoethanol, glycerol, and dye, and then boiled or heated at 68°C. The *S. aureus* was removed by centrifugation, and the supernatants were analyzed by electrophoresis through sodium dodecyl sulfate-polyacrylamide slab gels, as described below.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Discontinuous polyacrylamide slab gels were used as described (6). Gels of 10% acrylamide and exponential gradient gels of 5 to 20% and 10 to 20% acrylamide were used most commonly. After staining and fixation, gels were either dried driectly or first treated for fluorography with dimethyl sulfoxide–2,5-diphenyloxazole (1) or with 1 M sodium salicylate. They were then exposed to XR5 X-ray film.

Electron microscopy of cells and virus. Cells were processed either as pellets or as adherent monolayers. As indicated below, they were sometimes washed with PBS or cacodylate buffer before fixation with 2% glutaraldehyde for 30 to 60 min at the temperature indicated in the text. They were then washed with PBS or cacodylate buffer, postfixed with osmium tetroxide, stained with uranyl acetate, and dehydrated in a graded series of alcohols. Samples were then embedded in an epon-araldite-dodecenylsuccinic anhydride resin. Thin sections were examined on a Philips 201 microscope after poststaining with uranyl acetate and lead citrate.

RESULTS

Cleavage of Pr65^{gag} in R-*ts*24-infected cells at the nonpermissive temperature. The processing of the *gag* precursor, Pr65^{gag}, occurs poorly, if at all, in cells infected with either R-*ts*24 or M-*ts*3 and maintained at 39°C (13, 14, 18). To examine whether the addition of external protease to intact cells could initiate proteolytic processing, proteins from treated and untreated cells were analyzed by electrophoresis of [^{35}S]methionine-labeled cell extracts that had been immunoprecipitated with appropriate sera. R-*ts*24-infected cells were grown at 32°C (permissive temperature), shifted to 39°C (restrictive temperature), and metabolically labeled for 4 h with [^{35}S]methionine. The cells were chased at 39°C with fresh 39°C serum-free medium for up to 20 min with or without added trypsin and then rapidly chilled and lysed. In the absence of protease, only Pr65^{gag} and Pr55^{gag} (a product of Pr65 cleavage) were precipitated from the cell lysate by anti-p30 serum; virtually no labeled protein was released into the medium (Fig. 1, lanes A1 and A2). If cells similarly labeled at 39°C were instead shifted to 32°C for the 20 min chase, Pr65, Pr55, Pr40 (another *gag*-related cleavage product), and mature p30 were seen in the cells and in virions recovered from the medium (Fig. 1, lanes D1 and D2). The majority of the immunoreactive protein was in virions. When cells were exposed to 20 μg of trypsin per ml during a 5-min chase in 39°C serum-free medium, two new polypeptides (30,000 to 45,000 molecular weight) became evident in the cells, but not the medium, by immunoprecipitation with anti-p30 serum (Fig. 1, lanes B1 and B2). If this 5-min treatment was followed by the addition of soybean trypsin inhibitor and a further 15-min chase at 39°C, these new polypeptides were decreased in yield and p30 was recovered from the cells and the supernatant (Fig. 1, lanes C1 and C2).

Great care was taken to standardize the technical details of this experiment because of the hazard of an accidental shift-down of temperature with these temperature-sensitive mutants. Passage schedule and cell density were uniform. All treatments were done in DME equilibrated with CO_2 to maintain the pH. Manipulations of 39°C cultures were performed on a warm tray in a warm room with prewarmed reagents. Under these conditions, there was little difficulty in maintaining the fully restrictive conditions throughout the 39°C experiments as measured by the absence of released reverse transcriptase-containing particles in the medium of control 39°C cultures.

External trypsin treatment of the cells appeared to be triggering the cleavage of Pr65^{gag}, and the pathway to p30 was being completed even after trypsin activity was inhibited. Several preparations of trypsin, including that routinely used for passaging cells, had the same effect. The extent of cleavage of Pr65^{gag} varied with the concentration of the trypsin (Fig. 2, lanes C2, D2, E2). The cleavage was at least partly specific for *gag*, as no change was seen in the *env* protein (Fig. 2, lanes C1, D1, E1). This lack of effect of trypsin treatment on the *env* product was also seen when R-*ts*24-infected fibroblasts were stained with *env*-specific antiserum and fluoresceinated antibody to immunoglobulin G and examined microscopically. The intensity of

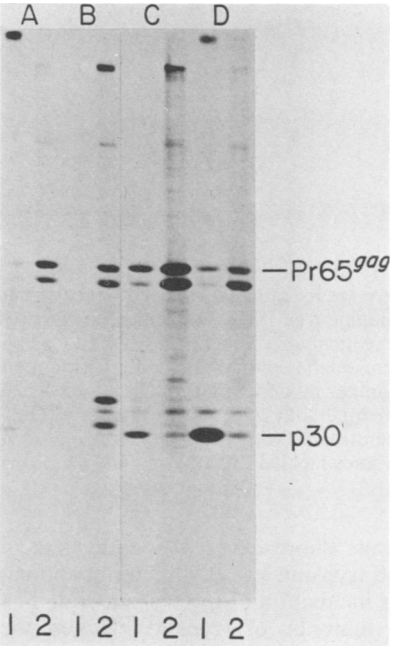

FIG. 1. Cleavage of Pr65^{gag} after treatment of intact cells with trypsin. R-*ts*24-infected 24N3 cells were seeded at 1.5 × 10^6 per 10-cm dish and grown for 2 days at 39°C. Cells were labeled for 4 h at 39°C in methionine-free DME supplemented with 100 μCi of [^{35}S]methionine. The labeling media were then discarded, cultures were rinsed with serum-free medium, and they were then incubated in fresh medium for an additional time under various conditions: (A) 20 min at 39°C, DME; (B) 5 min at 39°C, DME with 20 μg of trypsin per ml; (C) 5 min at 39°C, DME with 20 μg of trypsin per ml followed by 15 min at 39°C in the presence of 100 μg of soybean trypsin inhibitor per ml; (D) 20 min at 32°C, DME. The cultures were then rapidly chilled, and soybean trypsin inhibitor was added to 20 μg/ml. Media (lanes labeled 1) and cells (lanes labeled 2) were separately harvested, diluted with phospholysis buffer, and immune precipitated with anti-*gag* antiserum. Immunoprecipitates were analyzed by electrophoresis through a 10% polyacrylamide gel.

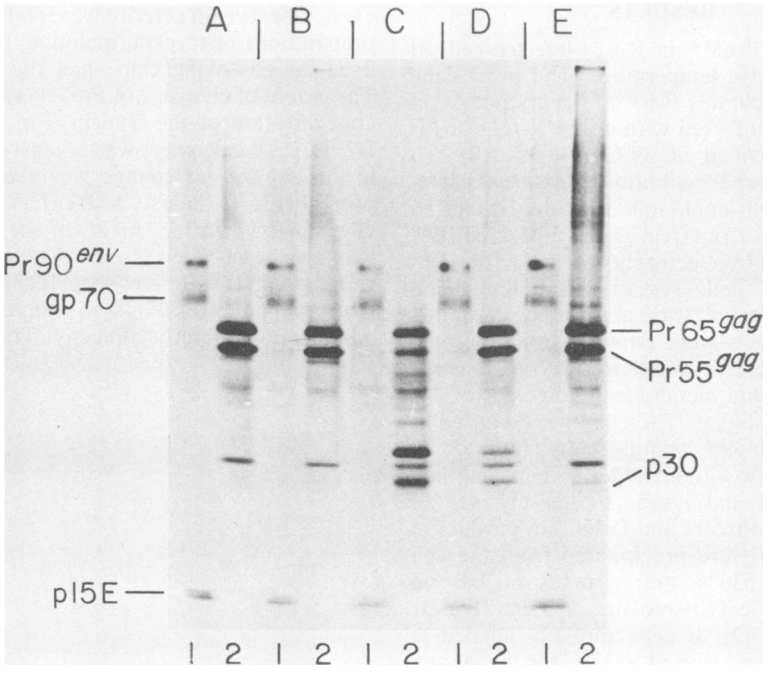

FIG. 2. Trypsin-induced cleavage of Pr65^{gag} is specific and concentration dependent. R-*ts*24-infected 24N3 cells were seeded at 1.5×10^6 per 10-cm dish and grown for 2 days at 39°C. Cells were then labeled for 2 h at 39°C in methionine-free DME supplemented with 100 μCi of [^{35}S]methionine per ml, complete DME was added, and cells were incubated for a further 2 h. The labeling medium was discarded, the cells were washed twice, and then they were incubated for 5 min under various conditions: (A) 39°C, PBS; (B) 39°C, PBS supplemented with 200 μg of trypsin per ml and 1 mM TLCK; (C) 39°C, PBS supplemented with 200 μg of trypsin per ml; (D) 39°C, PBS supplemented with 20 μg of trypsin per ml; (E) 39°C, PBS supplemented with 2 μg of trypsin per ml. The cells were then treated with 1 mM TLCK, rapidly chilled, lysed, and immunoprecipitated with (lanes labeled 1) anti-*env* or (lanes labeled 2) anti-*gag* antisera. Samples were analyzed on a 5- to -20% polyacrylamide gel.

membrane fluorescence was equivalent in control and trypsinized cells (data not shown).

Prior incubation of the trypsin with TLCK (a potent inhibitor of trypsin) blocked the *gag* cleavage (Fig. 2, lane B2). Incubations of 20 min at 39°C in the presence of increasing concentrations of trypsin gave higher intracellular yields of the new intermediates and p30. A greater yield of p30 in released particles was achieved, however, with milder and shorter treatments, implying that trypsin can damage the ability to release buds but still cause cleavage of the *gag* precursor proteins (data not shown).

The new *gag* gene intermediates appearing in the trypsin-treated cells corresponded to intermediates seen at very early times after shift-down of cultures to 32°C (data not shown; these bands are not apparent in Fig. 1, which displays the profile at 20 min after shift-down). The trypsin-induced pattern was thus consistent with physiological cleavage of the Pr65^{gag} precursor.

A similar induction of Pr65^{gag} cleavage at 39°C was also seen after treatment with chymotrypsin and thermolysin (but not with plasmin). The pattern of bands precipitated with anti-p30 serum was similar to that seen after trypsinization, although the relative intensities of the new bands were variable (data not shown). Trypsin cleaves after basic residues (lysine and arginine), chymotrypsin cleaves after aromatic residues (phenylalanine and tryptophan), and the specificity of thermolysin is not well characterized. Pr65^{gag} may present highly susceptible sites which can be cleaved by a variety of proteases, but the effectiveness of three dissimilar enzymes in inducing this cleavage makes it as likely that the added enzyme is inducing Pr65^{gag} cleavage indirectly. Activation of a second enzyme, or an indirect effect on a structural element, are likely mechanisms for this phenomenon.

Pr180$^{gag\text{-}pol}$ cleavage in R-*ts*24-infected cells at the nonpermissive temperature. Mature reverse transcriptase, p85, is derived by cleavage from a 180,000-dalton *gag-pol* precursor, with intermediates of 130,000 and 145,000 daltons; significant cleavage to p85 occurs only after virions have been released (18). Polymerase activity has been

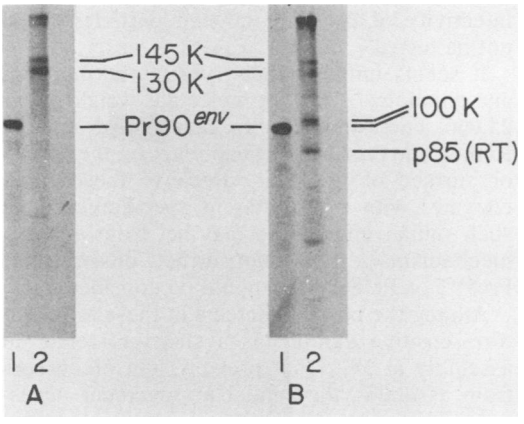

FIG. 3. Effect of trypsin treatment on *gag-pol* maturation. R-*ts*24-infected 24N3 cells were seeded at 1.5 × 10^6 per 10-cm dish and grown for 2 days at 39°C. Cells were then labeled for 4 h at 39°C in methionine-free DME with 50 μCi of [^{35}S]methionine per ml. Cells were then washed and incubated for an additional 30 min at 39°C after being refed with (A) DME, or (B) DME with 20 μg of trypsin per ml. Cells were lysed and immune precipitated with (lanes labeled 1) anti-*env* or (lanes labeled 2) anti-*pol* sera. Samples were analyzed on a 5 to 20% polyacrylamide gel.

detected in the mature p85 but not in the precursor or intermediate molecules (18). To examine $Pr180^{gag\text{-}pol}$ processing after protease treatment, R-*ts*24-infected cultures were shifted to 39°C and labeled for 4 h with [^{35}S]methionine. Cells were chased for 20 min at 39°C in fresh, unlabeled 39°C medium and then rapidly chilled and lysed. Intracellular polypeptides of molecular weight 180,000, 145,000, and 130,000 were precipitated with anti-reverse transcriptase serum (Fig. 3, lane A2). If a 20-min chase was performed with fresh medium containing trypsin, additional polypeptides were precipitated: a doublet with a molecular weight of approximately 100,000 and a band comigrating with mature reverse transcriptase at 85,000 daltons (Fig. 3, lane B2). Trypsinization apparently relieved blocks to both $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ cleavage in R-*ts*24-infected cells at 39°C.

Release of sedimentable reverse transcriptase activity. To examine the appearance of enzymatically active reverse transcriptase and the state of the viral proteins released upon trypsin treatment, we examined the supernatants of trypsin-treated cells for virions as measured by sedimentable reverse transcriptase activity. R-*ts*24-infected cells were shifted to 39°C for 4 h, the medium was changed, and the cells were either maintained at 39°C or shifted to 32°C. After 30 or 60 min, supernatants were chilled, and the particles sedimented by ultracentrifugation were assayed for reverse transcriptase activity. The 39°C collections repeatedly had low or background enzymatic activity. Cultures shifted to 32°C released a burst of virus, and enzyme assays often showed a 50-fold increase in reverse transcriptase activity above the 39°C control (Table 1). If the cultures were maintained at 39°C but the 30- or 60-min incubations were done in the presence of trypsin, high levels of reverse transcriptase were found in the supernatants (Table 1). Culture fluids collected in the presence of 5 μg of trypsin per ml had more enzyme activity than those treated with 20 μg of trypsin per ml. Both chymotrypsin and thermolysin were effective in increasing the level of release of reverse transcriptase-containing particles (Table 1).

In all experiments, comparable positive results were found in trypsin-treated cultures and cultures shifted to 32°C, but the magnitude of the released bursts varied. Attempts to standardize variables of pH, cell density, and cell passage

TABLE 1. Effect of temperature and protease treatment on reverse transcriptase release by R-*ts*24-infected cells

Treatment of monolayers[a]	Reverse transcriptase activity (cpm × 10^3)[b]			
	Expt 1	Expt 2	Expt 3	Expt 4
Maintained at 39°C	7.0	0.8	2.0	0.5
Maintained at 39°C, with 20 μg of trypsin per ml	114.1	41.6		5.3
Maintained at 39°C, with 20 μg of thermolysin per ml			16.9	6.2
Maintained at 39°C, with 20 μg of chymotrypsin per ml				2.9
Shifted to 32°C	138.2	94.0	9.8	4.7

[a] 24N3 cells were seeded at 1.5 × 10^6 per 10-cm dish and grown for 2 days at 39°C. The cultures were then rinsed and refed with prewarmed DME and incubated for an additional 30 min under the conditions described above.

[b] The freshly added medium was harvested after 30 min and rapidly chilled. The virions were purified by sedimentation and then assayed for reverse transcriptase activity by using exogenously added template and primer, as described in the text. Activity was monitored by measuring the incorporation of [^{3}H]TTP into acid-precipitable material.

schedule did not eliminate the variablity in eliciting virion release.

Morphology of R-*ts*24-infected cells. The morphology of cultures infected with these temperature-sensitive mutants and maintained at 39°C has previously been studied (2, 18, 19, 21). The number of viral buds observed has been shown to vary with several parameters, including whether cultures are washed with buffer before fixation and the time elapsed between seeding the cultures and their fixation. With these points in mind, when we compared the morphologies of R-*ts*24-infected 24N3 cultures maintained at 39°C, shifted briefly to 32°C, or treated with protease at 39°C, all conditions were maintained as constant as possible. Importantly, all cultures were rinsed an equal number of times with growth medium whether they were treated with enzyme or not. Cultures held at 39°C displayed large numbers of late-stage buds on the cell membrane and very few complete, extracellular virions (Fig. 4A). In cultures shifted to 32°C for 30 min, the number of buds was far lower, and the number of free virions associated with the cell pellet was greatly increased (Fig. 4C). Cultures treated at 39°C with trypsin for 30 min had many floating or only loosely adherent cells. The cells were viable, however, and displayed a large number of processes. Buds were still evident on the outer cell membrane, but many free particles indistinguishable from virions were also observed (Fig. 4B). Most particles had the collapsed structure which correlates with mature *gag* proteins, rather than the sharp concentric ring characteristic of buds and immature particles. In other experiments, culture fluids were subjected to ultracentrifugation, and the trypsin-induced release from cells of particles with mature virion morphology was demonstrated.

DISCUSSION

In cells infected with the temperature-sensitive mutants R-*ts*24 and M-*ts*3, the coupled processes of proteolytic cleavage of precursors to mature proteins and virion release are blocked at 39°C. We showed that protease added externally to intact cells can mimic the effect of a shift to 32°C, phenotypically bypassing the temperature-sensitive block. Trypsin induced cleavage of $Pr65^{gag}$ to several intermediates and p30, and of $Pr180^{gag\text{-}pol}$ to intermediates and p85 at the nonpermissive temperature. We recovered sedimentable material from the supernatants of protease-treated, 39°C cultures with high levels of reverse transcriptase activity. Particles indistinguishable from virions were seen in the electron microscope in cultures treated with protease at 39°C. Protease treatment destroyed the infectivity of authentic virions, and therefore the infectivity of protease-induced particles could not be tested.

It seems unlikely that trypsin, thermolysin, and chymotrypsin, with molecular weights near 25,000, enter the cells to cause these effects. Rather, the enzymes probably act on the exterior surface of the cells. Because these three enzymes with very different specificities have such similar effects and may act from outside, mechanisms other than direct cleavage of $Pr65^{gag}$ or $Pr180^{gag\text{-}pol}$ should be considered.

Among the possible defects in these temperature-sensitive mutants is a structural error in assembly at 39°C that prevents the precursors from assuming the spatial arrangement necessary to initiate bud release and protein cleavage. Trypsin might then be acting to release a "stress" in configuration of the 39°C bud, allowing maturation to proceed normally. This could occur by cleavage of proteins on the cell surface or of structural elements within the membrane. Trypsin causes 24N3 cells to detach from the culture dish surface and round up, and the consequent changes in cytoskeletal structure might affect the bud configuration. This suggestion predicts that other agents affecting cell shape might have a similar effect on virus maturation. Cytochalasin, a cytoskeletal disrupter, did not, however, have an effect like that of trypsin, although the cells were made to round up and float (data not shown). Hypo- or hypertonicity also appeared to be ineffective in releasing the temperature-sensitive block (data not shown). These mutants are also temperature-sensitive in lymphoid cells which naturally grow as nonadherent cells (data not shown). We therefore consider it unlikely that the proteases act through such effects.

Another possible explanation of these morphogenetic mutants is that they could have a defect in one of the polyproteins that prevents its cleavage. It could be, for instance, a protease precursor which, in its mutant form, cannot begin a cascade of proteolysis. External protease treatment might somehow cleave an accessible bond and begin a cascade leading to a full complement of cleaved proteins. It is also possible that the mutants make a protease that is temperature sensitive in its activity, but it is not clear how an externally applied protease could produce all of the cleavages needed to form complete virion-like particles containing active reverse transcriptase.

The enzymes of cellular or viral origin that are responsible for these viral cleavages are as yet unknown. In the avian retroviral system, a virally coded thiol protease (p15) has been implicated in viral maturation (3, 9, 15). In the murine system, there have been reports of a 24,000-molecular-weight serine protease recoverable in

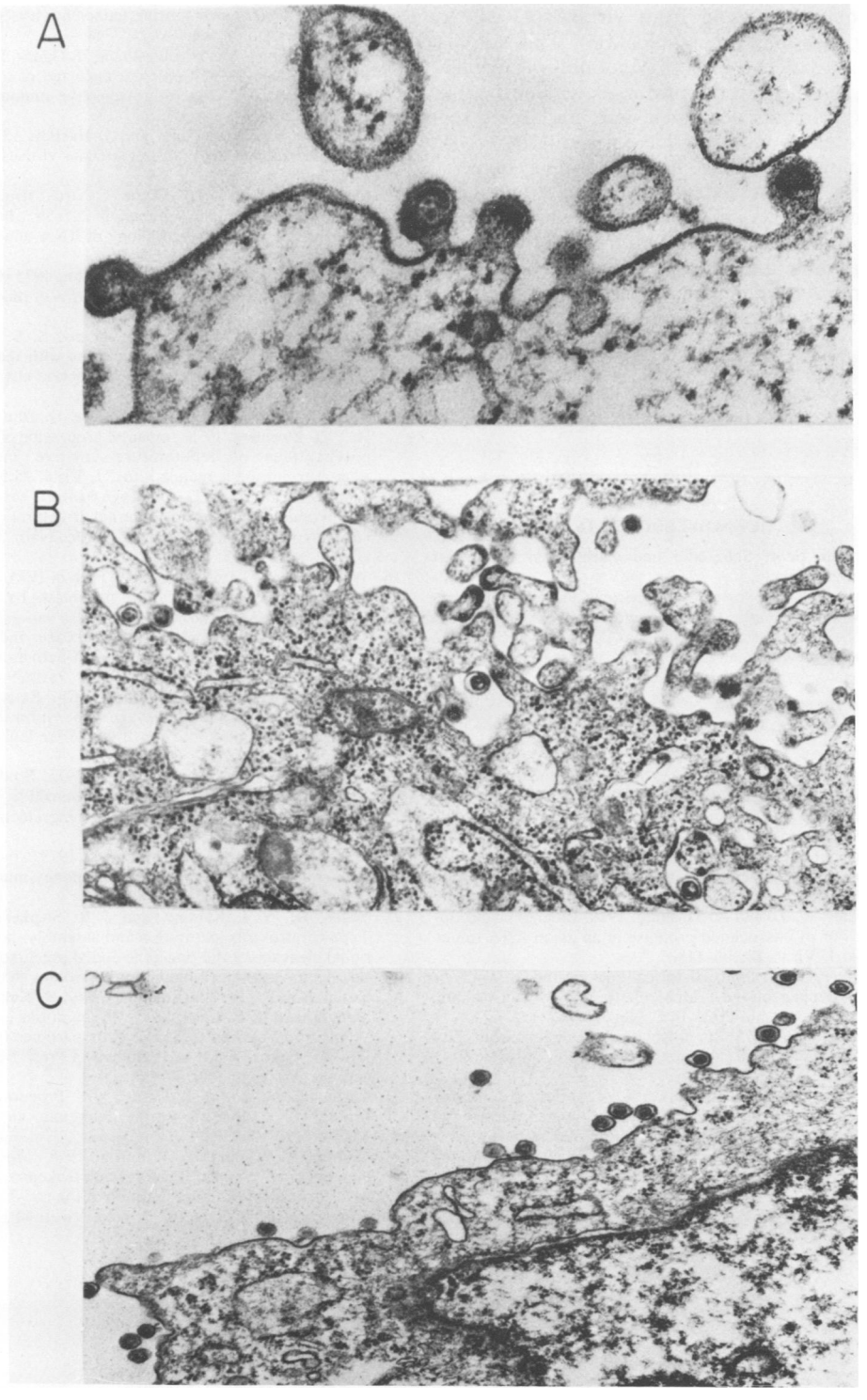

FIG. 4. Electron micrographs of R-*ts*24-infected 24N3 cells. R-*ts*24-infected 24N3 cells were seeded at 1.5×10^6 per 10-cm dish and grown at 39°C for 2 days. The cultures were then refed and incubated for an additional 20 min under the following conditions: (A) 39°C, DME (× 70,000); (B) 39°C, DME at 20 μg of trypsin per ml (× 30,000); (C) 32°C, DME (× 30,000). Cultures were then rinsed with buffer, fixed, and embedded. Representative micrographs are shown.

extremely low yield from virions (22–24; R. Luftig, personal communication). This activity appears to cleave $Pr65^{gag}$ accurately. It is interesting to note that the proteases with activity in the experiments described here are largely serine proteases and may have a similarity to the physiological protease in the murine system.

There are precedents in the literature for profound effects on cellular events mediated by external proteases. For instance, activation of membrane-bound adenyl cyclase by external trypsin has been demonstrated (16), and protease treatment can induce cells to produce and secrete collagenase and plasminogen activator (17). How the effects we have noted or the effects previously demonstrated are produced by an external protease remains an intriguing puzzle.

ACKNOWLEDGMENTS

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